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Mechanisms by Which *Candida albicans* Induces Endothelial Cell Prostaglandin Synthesis

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One strategy for improving resistance to opportunistic pathogens is to determine host cellular responses during the invasion process and upregulate those responses that are relevant to host defense mechanisms. Within this context, we have shown previously that invasion of endothelial cells by *Candida albicans* in vitro causes increased production of prostaglandins. As a prerequisite for modulating endothelial cell prostaglandin production, we now characterize the mechanisms through which this process occurs. Endothelial cell invasion by *C. albicans* appeared to stimulate the conversion of arachidonic acid into prostaglandins by upregulating the synthesis of endothelial cell cyclooxygenase and increasing the activity of the endothelial cell phospholipase. The enhanced activities of these two enzymes were independent of calphostin C-sensitive protein kinase C and resulted in the increased production and extracellular secretion of prostaglandin I_2 (PGI_2), $PGF_{2\alpha}$, and PGE_2. The secretion of these prostaglandins had no effect on the amount of endothelial cell injury induced by *C. albicans*. The role of the increased prostaglandin secretion by endothelial cells is likely related to modulation of the leukocyte response at the candida-leukocyte-endothelial cell interface.

During the evolution of disseminated candidiasis, hematogenous seeding of *Candida albicans* leads to the development of widespread microabscesses. To egress from the vasculature and enter the tissue parenchyma, it is likely that blood-borne fungi must first bind to, and then traverse, endothelial cells. Thus, endothelial cells may be among the first host cells to interact with these organisms as they invade. In addition to serving as the target of candidal adherence and penetration, it is highly probable that endothelial cells also alter the host inflammatory response to these organisms. By releasing eicosanoids (16) and cytokines (21) into the local environment or via the surface expression of platelet-activating factor (22) and various leukocyte adhesion molecules (31), endothelial cells can modulate the function of other immune effector cells such as neutrophils, monocytes, and lymphocytes.

C. albicans (11), as well as other microbial pathogens including *Rickettsia prowazekii* (32), *Staphylococcus aureus* (9), *Treponema pallidum* (26), and cytomegalovirus (30), has been shown to stimulate endothelial cells to express or release substances that modulate the function of leukocytes. However, the intracellular events that occur in endothelial cells as they respond to infectious agents have not been well characterized. For example, the mechanisms that mediate endothelial cell prostaglandin synthesis have been studied mainly by using soluble stimuli such as interleukin 1β (1), calcium ionophores (5), and thrombin (18). However, analogously to the situation in neutrophils (20), the response of endothelial cells to microorganisms may be different from their response to soluble stimuli. In the current study, we used *C. albicans* as the stimulus to elucidate the mechanisms by which endothelial cells respond to contact with blood-borne microbial pathogens.

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Previously, we showed that *C. albicans* stimulated endothelial cells to synthesize and release eicosanoids, mainly prostaglandin I_2 (PGI_2), in vitro (11). Since prostaglandins are known to be important regulators of the inflammatory response, including neutrophil function, hemostasis, and vascular tone, we defined the mechanisms by which *C. albicans* stimulated endothelial cells to synthesize and release prostaglandins.

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MATERIALS AND METHODS

Reagents. Yeast nitrogen base broth was purchased from Difco (Detroit, Mich.), and Hanks balanced salt solution (HBSS) with calcium and magnesium and RPMI 1640 were obtained from Irvine Scientific (Santa Ana, Calif.). Fetal bovine serum and medium 199 (M-199) were acquired from Gibco (Grand Island, N.Y.), and defined bovine calf serum was acquired from HyClone (Logan, Utah). The collagen matrix (Vitrogen) was bought from Celtrix (Palo Alto, Calif.), and calphostin C was from Calbiochem (San Diego, Calif.). 6-Keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and antiserum to each of these prostaglandins were purchased from Advanced Magnetics Inc. (Cambridge, Mass.). [^{3}H]6-keto-$PGF_{1\alpha}$ and [^{14}C]arachidonic acid were obtained from New England Nuclear (Boston, Mass.). Arachidonic acid, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2 standards were purchased from Biomol Research Laboratories (Plymouth Meeting, Pa.). High-performance liquid chromatography (HPLC) solvents (optima grade) were obtained from Fisher Scientific (Santa Clara, Calif.). All other reagents were purchased from Sigma (St. Louis, Mo.). The calphostin C, phorbol myristate acetate (PMA), and acetylsalicylic acid (ASA) were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was less than 0.07% (vol/vol) in all

experiments. In preliminary experiments, we determined that this concentration had no effect on endothelial cell injury or on the release of PGI_2.

Organisms. *C. albicans* 36082 was obtained from the American Type Culture Collection (Rockville, Md.). *Candida tropicalis* 5468 was a clinical isolate obtained from the clinical laboratory at Harbor-UCLA Medical Center. The wild-type strain, *C. albicans* SC5314, and three mutants, SC15183, SC15184, and SC15185, that were deficient in extracellular phospholipase activity were generous gifts from Daniel Bonner (Bristol-Myers Squibb, Wallingford, Conn.) (23). Growth and harvesting of the organisms were as previously outlined (11). Singlet blastospores were counted with a hemacytometer and adjusted to the desired concentration in either HBSS or RPMI 1640.

Determination of candidal phospholipase activity. Each organism was screened for production of extracellular phospholipases by growing it on egg yolk agar and measuring the size of the zone of precipitation by the method of Price et al. (25) as modified by Samaranayake et al. (27). Organisms which secrete extracellular phospholipases produce a zone of precipitation around individual colonies. In all experiments, organisms with and without phospholipase activity (*C. albicans* 36082 and *C. tropicalis* 5468, respectively) were tested concurrently. Using this method, we confirmed the findings of Minassian et al. (23) that *C. albicans* SC5314 produced extracellular phospholipases, whereas the mutants SC15183, SC15184, and SC15185 did not.

Endothelial cells. Human umbilical vein endothelial cells were prepared by our standard protocol with a modification of the method of Jaffe et al. (19). They were harvested in collagenase and grown in M-199 supplemented with 10% fetal bovine serum and 10% defined bovine calf serum. Second- or third-passage cells were grown to confluency either in 24-well tissue culture plates (Costar, Van Nuys, Calif.) coated with a collagen matrix or in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) coated with 0.2% gelatin. All incubations were in 5% CO_2 at 37°C unless otherwise noted. The cells were given fresh growth medium the day before each experiment.

Endothelial cell damage. The degree of endothelial cell injury caused by *C. albicans* and the various pharmacologic agents was determined by measuring the release of ^{51}Cr as described previously (11, 13). The specific release of ^{51}Cr induced by the different stimuli was calculated by the formula (experimental release − spontaneous release)/(total incorporation − spontaneous release).

Determining the effects of agonists and inhibitors on the endothelial cell response to *C. albicans*. The effects of various agonists and inhibitors on *Candida*-induced endothelial cell damage and release of PGI_2 were examined by using endothelial cells grown in 24-well tissue culture plates. In some experiments, the endothelial cells were exposed to 10 μM ASA for 30 min and then rinsed twice prior to exposure to the organisms. In other experiments, the cells were treated with 100 nM PMA for 10 min and then washed prior to exposure to *C. albicans*. However, when calphostin C (25 nM) was used, the endothelial cells were preincubated with this inhibitor for 30 min before, as well as during, exposure to various stimuli. In these experiments, the endothelial cells were placed under fluorescent lights (in ambient CO_2) for the first 20 min of incubation to activate the calphostin C (2). All other incubations were performed in the dark. At the concentrations used, none of these substances caused detectable endothelial cell injury as determined by release of ^{51}Cr. After initial exposure to the inhibitor or agonist, the wells of endothelial cells were overlaid with a suspension of 10^6 *C. albicans* blastospores in HBSS and the organisms were allowed to settle onto the monolayers by gravity. For experiments designed to measure the release of prostaglandins, parallel wells of endothelial cells were stimulated with the calcium ionophore A23187 (0.5 μM) as a positive control. In previous experiments (11), this concentration of ionophore was not toxic to the endothelial cells as measured by release of ^{51}Cr. After the plates were incubated for 2 or 5 h, the contents of the wells were aspirated and processed as described below. All conditions were tested in triplicate or quadruplicate.

Measurement of PGI_2. The conditioned media from the 24-well plates were centrifuged at 11,000 × *g* for 5 min to remove particulate matter, and the supernatants were stored at −20°C for later analysis. The concentration of PGI_2 in the supernatants was quantified by measuring its stable metabolite, 6-keto-$PGF_{1\alpha}$, by using a radioimmunoassay as previously described (11).

HPLC. Confluent monolayers of endothelial cells in 75-cm^2 tissue-culture flasks were used to investigate which prostaglandins were synthesized by the endothelial cells from exogenous arachidonic acid. On the day of the experiment, the growth medium was aspirated and the endothelial cells were rinsed twice with warm HBSS. Next, 12 ml of HBSS with or without 3×10^6 *C. albicans* 36082 cells per ml was added, and the cells were incubated. After 2 h, the medium in each flask was replaced with 12 ml of HBSS containing 0.6 μCi of [^{14}C]arachidonic acid (10 mCi/mmol) and the cells were incubated for an additional 1 h. At the end of incubation, the liquid medium in each flask was aspirated, cooled on ice, and then centrifuged at 1,000 × *g* for 10 min at 4°C. Next, the supernatants were collected and stored at −20°C for later analysis.

To estimate the relative amounts of PGI_2, $PGF_{2\alpha}$, and PGE_2 produced by endothelial cells, the samples were acidified with glacial acetic acid to pH 3 and the prostaglandins were extracted on Bakerbond C_{18} columns (J. T. Baker, Phillipsburg, N.J.) (3). After evaporation under nitrogen, the samples were redissolved in 0.2 ml of 31% acetonitrile for analysis by HPLC on a Nucleosil C_{18} column (5 μm, 4.6 by 250 mm; Phenomenex, Torrance, Calif.). The samples were eluted isocratically at a flow rate of 1 ml/min by using a solvent system of 31% acetonitrile and 0.1% acetic acid in water. One-milliliter fractions were collected, and the amount of radioactivity in each fraction was measured by scintillation counting. The identities of the various peaks of radioactivity were determined by comparing their elution times with those of authentic standards.

Statistical analysis. The results of similar experiments performed on different days were evaluated by using a two-way analysis of variance with the Bonferroni correction for multiple comparisons. *P* values of <0.05 were considered significant. All results are expressed as means ± standard deviations.

RESULTS

***C. albicans* stimulates synthesis of prostaglandins by endothelial cells.** Exposure to *C. albicans* stimulated endothelial cells to synthesize prostaglandins from exogenous arachidonic acid (Fig. 1). In these experiments, the blastospores were observed to settle rapidly onto the endothelial cell monolayers, where they germinated within approximately 45 min. The majority of these germinated organisms were adherent to the monolayers, since replacing the medium with fresh HBSS that contained radiolabelled arachidonic acid did not decrease the number of organisms on the monolayer as determined by visual inspection using phase-contrast microscopy. As indi-

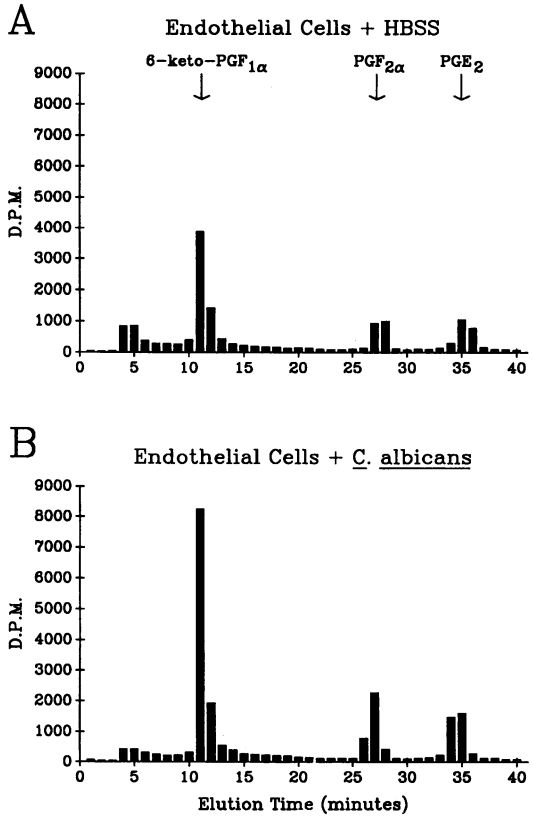

FIG. 1. Exposure to *C. albicans* stimulates endothelial cells to synthesize prostaglandins from exogenous arachidonic acid. Endothelial cells were exposed to buffer (A) or to *C. albicans* 36082 (B) for 2 h and then incubated with [^{14}C]arachidonic acid for an additional 1 h. The media were extracted for analysis by HPLC, and the amount of radiolabel incorporated into each fraction was determined. Elution times of authentic standards are shown at the top of the figure. Results, in disintegrations per minute, are representative of three experiments.

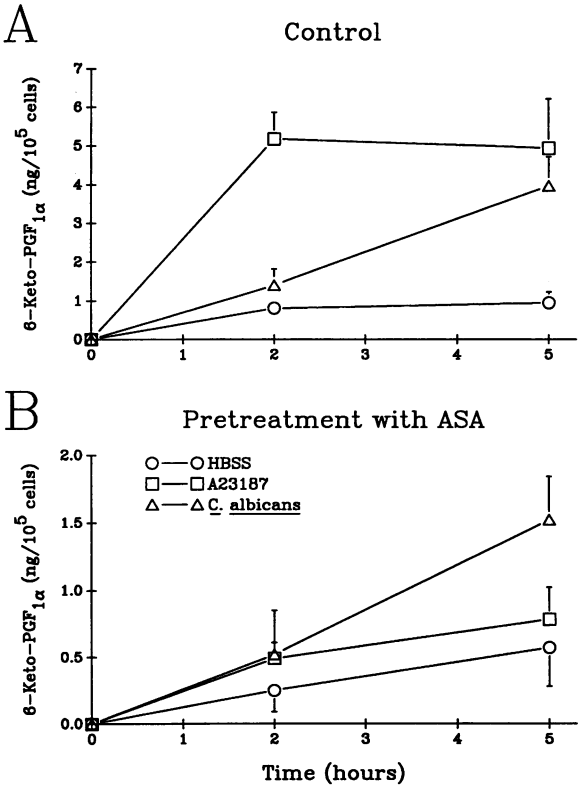

FIG. 2. *C. albicans* enhances the rate of recovery from ASA. Endothelial cells were incubated with either HBSS (A) or ASA (B), rinsed, and then exposed to HBSS, calcium ionophore A23187, or *C. albicans* 36082 for the indicated times. The concentrations of PGI_2 in the medium were determined by radioimmunoassay for its stable metabolite, 6-keto-$PGF_{1\alpha}$. Results are means ± standard deviations (bars) of three experiments.

cated in Fig. 1A and B, contact with *C. albicans* stimulated the synthesis of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$), $PGF_{2\alpha}$, and PGE_2. These prostaglandins were synthesized by the endothelial cells and not by the organisms, since no detectable prostaglandins were synthesized when germinated *C. albicans* was incubated with [^{14}C]arachidonic acid in the absence of endothelial cells (data not shown).

Exposure to *C. albicans* increases the synthesis of endothelial cell cyclooxygenase. Because exposure to *C. albicans* caused endothelial cells to increase synthesis of three different prostaglandins from exogenous arachidonic acid, we examined the possibility that the organisms stimulated the enhanced production of prostaglandins by increasing the activity of cyclooxygenase. As indicated in Fig. 2, blocking endothelial cell cyclooxygenase activity by pretreatment with ASA significantly inhibited the release of PGI_2 in response to buffer, *C. albicans*, and the calcium ionophore ($P < 0.03$ for each condition at each time point). Five hours after treatment with ASA, the endothelial cells exposed to *C. albicans* released almost twofold more PGI_2 than did the cells stimulated with the calcium ionophore ($P < 0.001$). This accelerated prostaglandin synthesis by the *Candida*-infected cells was evidence that the organisms stimulated the de novo synthesis of cyclooxygenase.

To ensure that the differences in effects on the stimulation of prostaglandin synthesis between *C. albicans* and the calcium ionophore were not due to a toxic effect of the ionophore, we incubated ASA-treated endothelial cells in HBSS for 4.5 h and then exposed them to the calcium ionophore for 30 min. Cells treated in this manner released 0.66 ± 0.07 ng of PGI_2 per 10^5 cells, an amount similar to that released by endothelial cells exposed to the calcium ionophore for the entire 5 h ($P > 0.5$).

Phospholipase-deficient organisms stimulate PGI_2 synthesis. When added to endothelial cells in RPMI 1640, the two phospholipase-producing strains of *C. albicans*, 36082 and SC5314, stimulated comparable PGI_2 release ($P < 0.5$; Fig. 3A). The three mutants, which were completely deficient in extracellular phospholipase production, induced 19 to 39% less release of PGI_2 than did strain 36082. This difference was statistically significant for strains SC15183 and SC15185 (P = 0.002 and 0.03, respectively), but not for strain SC15184 (P = 0.09). Since the phospholipase-deficient mutants and the parent strain were still able to stimulate endothelial cells to synthesize and release PGI_2, the endothelial cell phospholipase, not the candidal phospholipase, must have been responsible for the enhanced mobilization of arachidonic acid and subsequent prostaglandin synthesis.

Effects of protein kinase C on prostaglandin synthesis. To determine whether *C. albicans* stimulated endothelial cells to synthesize prostaglandins via a protein kinase C-dependent mechanism, we pretreated the endothelial cells with calphostin C (an inhibitor of protein kinase C) and/or PMA (a protein

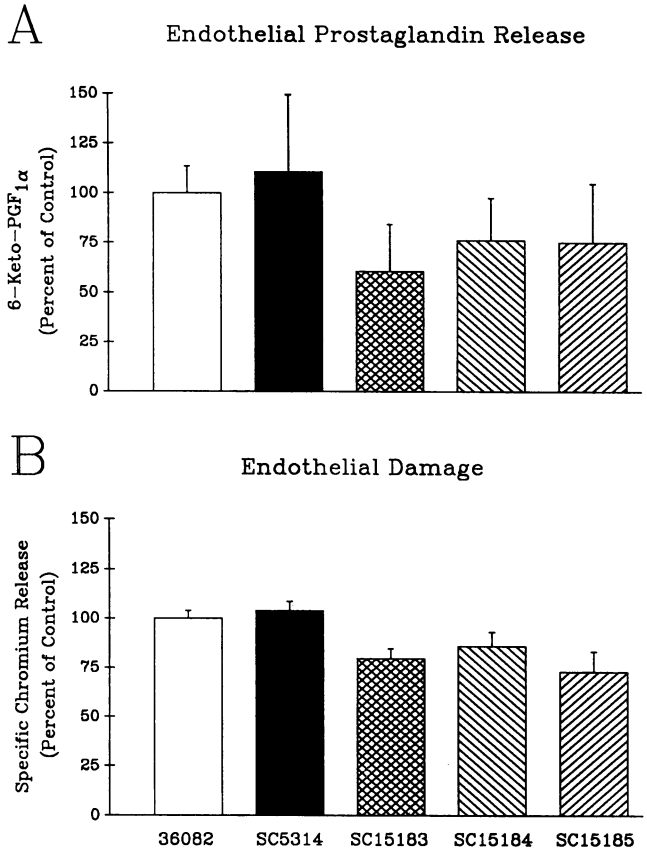

FIG. 3. Ability of mutants of *C. albicans* that were deficient in extracellular phospholipase activity to induce the synthesis of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) (A) and damage endothelial cells (determined by release of ^{51}Cr) (B). Results are expressed as the percentages of response induced by strain 36082 and are means + standard deviations (error bars) of three experiments. The release of 6-keto-$PGF_{1\alpha}$ stimulated by 36082 was 3.8 to 6.3 ng/10^5 cells, and the specific release of ^{51}Cr was 50 to 64%.

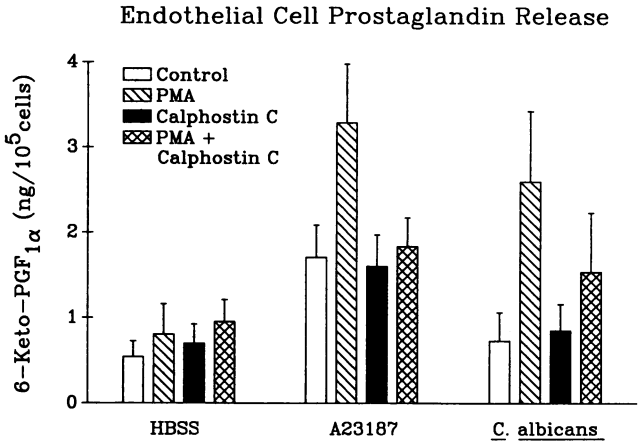

FIG. 4. Effects of protein kinase C activation and inhibition on endothelial cell synthesis of PGI_2. Endothelial cells were exposed to HBSS, PMA, calphostin C, or both PMA and calphostin C and then incubated with HBSS, the calcium ionophore (A23187), or *C. albicans* 36082 for 2 h. The concentration of PGI_2 in the medium was measured as 6-keto-$PGF_{1\alpha}$ (means + standard deviations [error bars] of three experiments).

kinase C activator) prior to stimulating them with either *C. albicans* or the calcium ionophore. In these experiments, a 2-h period of stimulation was used because exposing the endothelial cells to calphostin C for greater than 2 h caused significant toxicity (data not shown).

As shown in Fig. 4, calphostin C had no effect on the quantity of PGI_2 that was released from unstimulated endothelial cells or from cells exposed to ionophore or *C. albicans*. The concentration of calphostin C used in these experiments was sufficient to block protein kinase C activation, since calphostin C significantly inhibited the stimulatory effects of PMA on PGI_2 production in response to the calcium ionophore and *C. albicans* ($P < 0.01$ for each stimulus). Thus, protein kinase C does not mediate the increase in prostaglandin synthesis caused by *C. albicans*.

Cyclooxygenase inhibition does not decrease endothelial cell damage. Inhibiting endothelial cell cyclooxygenase activity with ASA had no effect on the degree of injury caused by *C. albicans*, even though this inhibitor decreased the release of PGI_2. At 5 h, the specific release of ^{51}Cr from endothelial cells infected with *C. albicans* was 36.5 ± 4.4% without ASA pretreatment and 36.8 ± 3.7% with ASA pretreatment ($P > 0.5$, $n = 3$). Therefore, the synthesis and release of prostaglandins by endothelial cells did not alter the degree of endothelial cell injury caused by candidal invasion.

Phospholipase-deficient mutants are able to damage endothelial cells. As shown in Fig. 3B, the phospholipase-producing strains, 36082 and SC5314, caused similar amounts of endothelial cell injury ($P > 0.5$). The phospholipase-deficient mutants, SC15183, SC15184, and SC15185, caused 17 to 30% less damage to the endothelial cells than did the parent strain ($P \leq 0.01$ for each organism).

Inhibiting protein kinase C blocks endothelial cell injury. Exposing endothelial cells to PMA did not significantly alter the extent of injury caused by *C. albicans* (11 ± 19% increase in damage; $P = 0.4$, $n = 3$); however, exposure to calphostin C decreased the amount of endothelial cell damage by 31 ± 15% ($P < 0.015$, $n = 3$). In other experiments, to ensure that the protective effect of calphostin C was not due to its action on the organisms we exposed endothelial cells to calphostin C for 30 min and then removed the inhibitor by washing prior to adding *C. albicans*. When used in this manner, calphostin C decreased *Candida*-induced endothelial cell injury by a similar amount (27 ± 18%; $P < 0.001$, $n = 7$).

DISCUSSION

We have shown previously that prostaglandins are the principal eicosanoids secreted by *Candida*-stimulated endothelial cells (11). In the current study, we found that exposure to *C. albicans* stimulated endothelial cells to synthesize mainly PGI_2 and lesser amounts of $PGF_{2\alpha}$ and PGE_2 (Fig. 1). However, relative to basal release, the synthesis of all three prostaglandins was increased by roughly the same factor (1.6- to 1.8-fold). This pattern of prostaglandin release is similar to that seen when endothelial cells are stimulated by thrombin or exogenous arachidonic acid (7). Because the synthesis of all three prostaglandins increased equally, it is probable that *C. albicans* enhanced the activity of cyclooxygenase, which catalyzes the synthesis of PGH_2, the common precursor of PGI_2, $PGF_{2\alpha}$, and PGE_2 (29), rather than stimulating the enzymes that catalyze the synthesis of each of the three prostaglandins separately.

To determine if *C. albicans* stimulated endothelial cells to synthesize cyclooxygenase, the rate at which endothelial cells recovered from the inhibitory effects of ASA was examined. Since endothelial cells that were infected with *C. albicans* recovered from the effects of ASA significantly faster than the controls, we conclude that the organism enhances the de novo synthesis of cyclooxygenase. Similar findings have been reported for interleukin 1β (1). Recently, it was discovered that human umbilical vein endothelial cells possessed two cyclooxygenase genes, *hCox-1* and *hCox-2* (15). The former gene was expressed constituitively whereas the latter gene was inducible. Although not specifically determined in these experiments, it seems probable that *C. albicans* stimulates cyclooxygenase synthesis by increasing the activity of *hCox-2*.

Previously, we found that exposure to *C. albicans* stimulated the mobilization of arachidonic acid from the phospholipids of endothelial cells (11). Arachidonic acid serves as the substrate from which prostaglandins are synthesized by cyclooxygenase (28). In the present study, we determined that mobilization of arachidonic acid was catalyzed by the phospholipases in the endothelial cells and not the candidal phospholipases. In endothelial cells, arachidonic acid release is likely catalyzed by phospholipase A_2 (4, 6), although we cannot completely rule out the possibility that *C. albicans* stimulated the sequential activities of phospholipase C and diglyceride lipase (28). Walker et al. (32) have reported that *R. prowazekii* also stimulated endothelial cells to release both PGI_2 and PGE_2. However, in contrast to the situation with *C. albicans*, it appears that the rickettsial phospholipase is required for mobilization of arachidonic acid and subsequent prostaglandin synthesis.

Because activation of protein kinase C increases prostaglandin synthesis in endothelial cells by both upregulating the activity of phospholipase A_2 (5, 12) and augmenting the synthesis of cyclooxygenase (33), we examined whether the effects of *C. albicans* on endothelial cells were mediated by this signal transduction mechanism. Although stimulating the activity of protein kinase C with PMA potentiated prostaglandin release induced by *C. albicans*, blocking the activity of this enzyme with calphostin C had no effect on the amount of prostaglandins secreted by the infected cells. Since calphostin C also had no effect on the synthesis of PGI_2 induced by *C. albicans*, this organism must stimulate arachidonic acid metabolism in endothelial cells by a mechanism that is either independent of protein kinase C or mediated by a form of protein kinase C that is insensitive to this inhibitor. For example, nuclear protein kinase C is insensitive to calphostin C and most other inhibitors of this enzyme (8). This form of protein kinase C has been found in neutrophils; however, it is not known if endothelial cells possess nuclear protein kinase C.

In previous studies, we found that the extent of endothelial cell injury and the release of PGI_2 induced by *Candida* species were closely associated (11). The strains and species of *Candida* that stimulated the highest release of PGI_2 also caused the most damage to the endothelial cells. In the current study, we used ASA and PMA to inhibit and stimulate, respectively, the synthesis of prostaglandins by endothelial cells that were infected with *C. albicans*. Neither of these pharmacologic agents altered the degree of endothelial cell injury caused by the organism. Therefore, we hypothesize that, in the absence of other inflammatory cells, prostaglandins do not modulate the degree of endothelial cell damage that occurs during candidal invasion. This conclusion is supported by our finding that the cyclooxygenase inhibitor indomethacin also had no effect on the degree of endothelial cell injury caused by *C. albicans* (data not shown).

In contrast to ASA and PMA, calphostin C did not affect prostaglandin release, but it did decrease the extent of *Candida*-induced endothelial cell injury by a significant amount. Therefore, in endothelial cells, the activity of protein kinase C partially determines the degree of damage that is caused by *C. albicans*, even though activation of this enzyme is not responsible for the increase in prostaglandin synthesis that occurs during candidal invasion. Our results also suggest that, in response to *C. albicans*, endothelial cell injury and prostaglandin release are independent processes that are controlled by different signal transduction mechanisms. At present, the precise mechanism by which *C. albicans* causes endothelial cell injury and the cytoprotective responses of endothelial cells to candidal invasion remain to be elucidated. However, characterizing these processes is critical to developing strategies to enhance the endothelial cell defense to hematogenously disseminated infections.

The release of eicosanoids by endothelial cells is known to regulate vascular tone (24), permeability (34), and thrombogenicity (17). However, these substances may also be important in modifying the local inflammatory response to microbial pathogens such as *C. albicans*. Possibly, the release of PGI_2 and PGE_2 by infected endothelial cells is detrimental to the host immune response, since these prostaglandins inhibit neutrophil function (14, 35). Alternatively, modulating neutrophil activity by the local production of prostaglandins may protect the endothelial cells from damage caused by activated phagocytes. As a possible example of this phenomenon, we have shown that neutrophils are able to phagocytize and kill *C. albicans* on endothelium without injuring the endothelial cells (10). Studies to investigate the role of prostaglandins in modulating the effects of activated neutrophils on *C. albicans* and endothelial cells are currently in progress in our laboratory.

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